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Evolution of Developmental Control Mechanisms

Cellular and molecular investigations into the development of the pectoral girdle

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ABSTRACT

The forelimbs of higher vertebrates are composed of two portions: the appendicular region (stylopod, zeugopod and autopod) and the less prominent proximal girdle elements (scapula and clavicle) that brace the limb to the main trunk axis.

We show that the formation of the muscles of the proximal limb occurs through two distinct mechanisms. The more superficial girdle muscles (pectoral and latissimus dorsi) develop by the "In–Out" mechanism whereby migration of myogenic cells from the somites into the limb bud is followed by their extension from the proximal limb bud out onto the thorax. In contrast, the deeper girdle muscles (e.g. rhomboideus profundus and serratus anterior) are induced by the forelimb field which promotes myotomal extension directly from the somites. *Tbx5* inactivation demonstrated its requirement for the development of all forelimb elements which include the skeletal elements, proximal and distal muscles as well as the sternum in mammals and the cleithrum of fish. Intriguingly, the formation of the diaphragm musculature is also dependent on the *Tbx5* programme. These observations challenge our classical views of the boundary between limb and trunk tissues. We suggest that significant structures located in the body should be considered as components of the forelimb.

Introduction

The limbs of higher vertebrates need to fulfil two quite distinct roles in order to function as a single unit; the appendicular portion distal to the shoulder joint is required to generate skeletal muscle contraction needed to mediate propulsion whereas the proximal (girdle) region is required to brace the limb to the axial skeleton in order to transmit propulsion to the body. The proximal limb (girdle) of the vertebrates underwent considerable re-enforcement following the evolutionary transition from aquatic to terrestrial life-style, which allowed the body to be lifted off the ground.

While the evolution and development of the distal appendicular limb and its outgrowth have been studied in considerable detail, there is a paucity of data explaining the development of the proximal limb, the relationship with the skeletal girdle and the associated musculature. Although the skeletal elements comprising the proximal pectoral girdle and the distal appendicular limb function as a single anatomical unit, they are made of cells from two distinct sources. Most of the limb skeleton originates from lateral plate mesoderm. However the scapular blade in birds and medial border of scapula in mammals are derived from the para-axially located somites (Huang et al., 2000; Valasek et al., 2010). The scapula blade is responsible for anchoring the girdle to the main body axis. Furthermore and pertinent to this study is the fact that the connective tissue needed to brace the proximal limb to the axial skeleton is continuous from the limb to the superficial tissues of the thoracic region. Therefore in order to form a functional forelimb capable of fulfilling the locomotor requirements of terrestrial animals, a developmental programme must be capable of coordinating the

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differentiation of tissues from a number of embryonic origins located along the medio-lateral axis.

The most prominent muscles of the pectoral girdle are the pectoral and serratus anterior muscles (ventrally) and the latissimus dorsi and deeper rhomboid muscles (dorsally). The anatomical attachments for the pectoralis muscles span from the sternum and the adjacent ribs to the proximal humerus and coracoid, and for the serratus anterior from the ribs to the medial scapula margin; for the latissimus dorsi from the spinous processes of the thoracic vertebrae to the proximal humerus, for the rhomboids again from the spinous processes of the cervical/thoracic vertebrae to the medial scapula margin (scapular blade in birds) allowing for scapular protraction and retraction, respectively.

We show the developmental processes responsible for the formation of all forelimb muscles and illustrate the involvement of three mechanisms: Direct migration into the forelimb to form classical limb non-girdle muscles, 'In–Out' mechanism to form the superficial girdle muscles and direct extension from the myotomes to form the deep girdle muscles. We show that the development of all muscles and all elements of the forelimb are controlled by the transcription factor Tbx5. Furthermore we propose an intimate relationship between the sternum, cleithrum and diaphragmatic musculature and the forelimb development programme.

Materials and methods

Embryos

Mouse embryos were staged according to Kaufman(1992). Noon on the day a vaginal plug was observed was taken to be 0.5 days post coitum. The mouse lines carrying a conditional allele of Tbx5 (Bruneau et al., 2001) and a Prx1Cre transgene (Logan et al., 2002) have been described previously. Fertilised chicken and quail eggs were incubated at 38 °C and 80% humidity and staged according to reference texts (Hamburger and Hamilton, 1951) and (Ainsworth et al., 2010).

Whole-mount in situ hybridisation

In situ hybridisation was performed according to Nieto et al. (1996). Briefly, chick embryos HH23–35 and mouse embryos were fixed in 4% PFA/PBS/0.1% Triton, dehydrated in methanol, re-hydrated, treated with proteinase K and re-fixed. For good quality in situ hybridisation, chicken HH32 embryos and older were skinned at this stage. Anti-sense RNA probes of chicken Tbx5 (1000 bp probe), mouse Tbx5 (Bruneau et al., 1999), chicken MyoD (1518 bp), mouse MyoD (1833 bp) and chick HoxB9 (400 bp) were labelled with digoxigenin. Fab fragments of sheep antibody against digoxigenin, conjugated to alkaline phosphatase, mediated visualisation (1:5000, Roche).

Chick/quail/GFP chimeras

Chick recipient embryos were manipulated in ovo (HH12–23). The donor tissue was isolated using electrolytically sharpened tungsten needles, transferred to the chick surgery site with a thin glass Pasteur pipette and positioned by drawn glass needles. Limb buds at HH23 were fixed in position by a cactus needle or closure of the amnion. 1–2 ml of albumin was removed, the egg was closed with surgical tape and re-incubated.

Immunohistochemistry

Embryos were fixed in 4% PFA/PBS (maximum of 2 h in case of GFP chimeras). Embryos were dehydrated in ethanol, xylene and embedded in paraffin. Transverse 10 μm serial sections were re-hydrated, boiled in 10 mM citrate pH 6.0 for antigen retrieval (for myosin heavy chain only), pre-blocked in 10% heat inactivated goat serum in PBS, incubated over-night with myosin heavy chain (DSHB A41025 supernatant 1:4) or quail nuclear antigen (DSHB QCPN, supernatant undiluted), which were detected with a secondary rabbit anti-mouse antibody conjugated to biotin (DAKO) and developed with ABC streptavidin/peroxidase kit and DAB staining (Vectorlabs). Sections were counterstained with Alcian blue (0.05% in 0.05% acetic acid). In case of double staining for GFP antigen, we used also anti-GFP rabbit polyclonal antibody (Torrey Pines Biolabs) and goat-anti rabbit 488 (Jackson Immuno Research) antibodies.

Zebrafish processing

Mutant *tbx5a*^{m21} (Garrity et al., 2002) and wild type embryos were maintained and staged as previously described (Westerfield, 1995). Alizarin red/Alcian blue staining and pan-skeletal MyHC (A4.1025 antibody) immunostaining was performed as previously described (Walker and Kimmel, 2007) (Hinits and Hughes, 2007).

Skeletal muscle nomenclature and photography

Identification of muscles was according to standard texts for avian muscles (Baumel et al., 1993; Nickel et al., 2004; Sullivan, 1962), for mouse muscles (Greene, 1935) and for human muscles (Williams et al., 1995). Whole embryos were photographed using a Nikon SMZ1500 stereomicroscope with a Nikon Coolpix digital camera, and sections using a Nikon Eclipse 400. Image processing was performed using Adobe Photoshop 5.0LE.

Results

Girdle musculature development

Delamination and migration of myogenic precursors from the somites into the limb bud and their subsequent patterning to form the appendicular limb muscles are controlled by cues from the limb bud mesoderm (Hayashi and Ozawa, 1995; Kardon, 1998). In contrast very little is known about the mechanisms controlling the patterning of the proximal limb girdle musculature.

We have recently discovered that the path of the myogenic cells from the somites to their final anatomical position in the caudal region of the embryo is not necessarily a direct one but via the hindlimbs (Valasek et al., 2005) — a process called the 'In–Out' mechanism (Evans et al., 2006). This mechanism is defined as the migration of myogenic precursors firstly from the somites into the developing limb bud. After a brief residential period in the limb bud, some of the myogenic precursors migrate out of the limb bud, back into the main body axis. Here we explored whether a similar mechanism was deployed in the forelimb region.

We found that during early stages of chick limb development (HH23) the limb pre-muscle masses were discontinuous with the trunk, with no evident cell/tissue extensions. However, subsequently, we found that the pre-muscle masses expanded 'Out' into the trunk to form the pectoral muscles ventrally and latissimus dorsi dorsally (Fig. 1). An identical situation was found in the mouse embryos (Fig. 1). These data suggested that the 'In–Out' mechanism was deployed in the forelimb region of birds and mammals.

In order to confirm that the pectoral and latissimus dorsi muscles, located in the trunk, had developed from a population that temporarily resided in the limb, we transplanted a compound wing bud consisting of GFP-chick myogenic cells and quail connective tissue (Fig. 2A). This experiment revealed two major features: Firstly, the superficial girdle muscles (e.g. pectoral and latissimus dorsi) were GFP-positive (Figs. 2B–D). In contrast, the deeper girdle muscles (rhomboids and the avian serrati) were GFP-negative. Thus only the superficial girdle muscles of the forelimb developed through the 'In–Out' mechanism. We have categorised these muscles as superficial and deep girdle muscles respectively not only to reflect their final

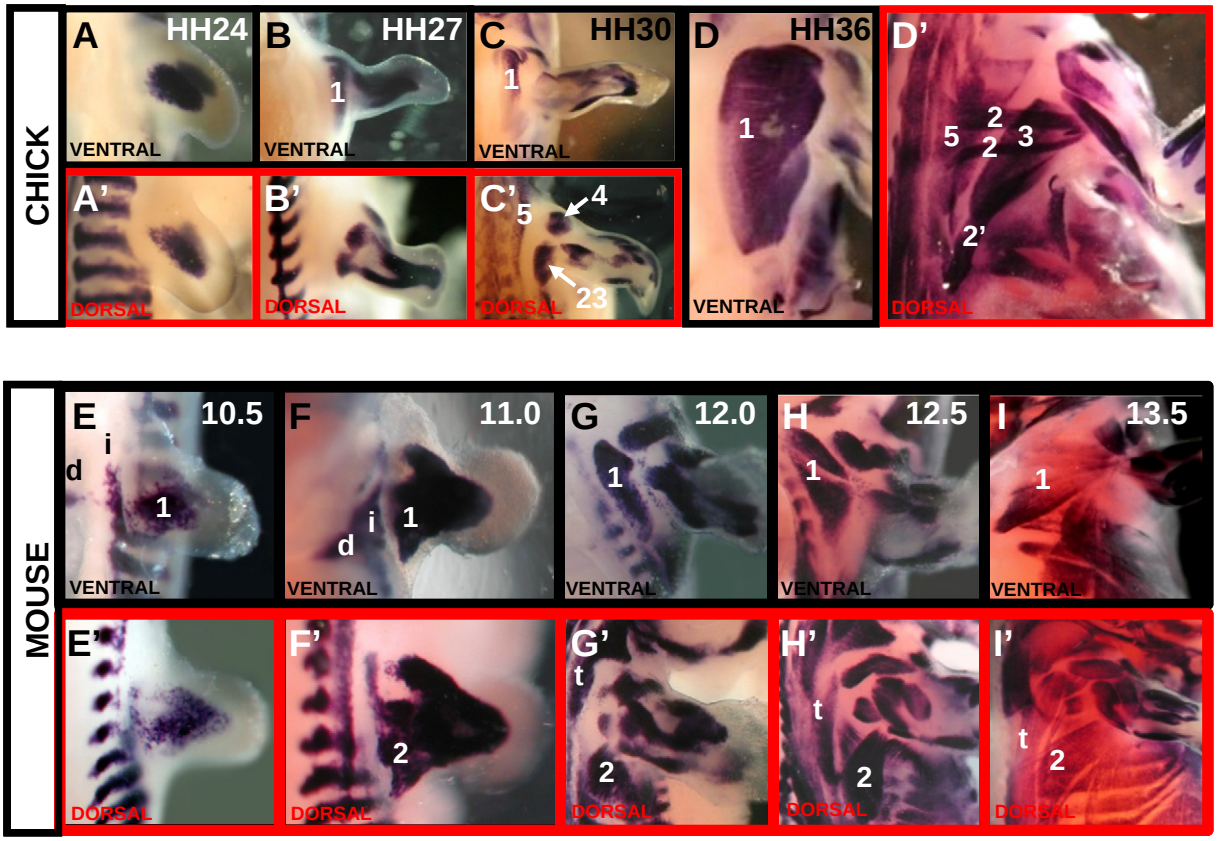

Fig. 1. "Out" phase of girdle muscle development. In-situ hybridisation for *MyoD* expression shows the "Out" phase of the ventral and dorsal pre-muscle masses in chick and mouse embryos, developing into pectoral and latissimus dorsi muscles respectively. The "In" phase — migration of myogenic cells from the somite to the limb bud has finished before HH23/10.5 dpc. (A–D) Chick embryos — ventral views showing the development of the pectoral muscle (1). (A′–D′) Mirror images of dorsal views document the development of dorsal pre-muscle mass into *latissimus dorsi* (*2—pars cranialis, 2′ pars caudalis*), *3—scapulohumeralis posterior*, *4—deltoideus*; (*5—rhomboid muscles* develop from axial myotomes). (E–I) 10.5–13.5 dpc mouse embryos show the extension of the ventral pre-muscle mass onto the thorax forming pectoral muscles (1). Other two muscle groups develop in the vicinity of the ventral aspect of the limb bud: d—diaphragm and i—intercostal muscles. (E′–I′) Mirror images of dorsal views document the development of *2—latissimus dorsi*; (*t—trapezius develops from the neck*).

anatomical position, but mainly their differing developmental source (from lateral source – the limb and from medial source – the myotome) and the temporal sequence of their attachment points to the future skeletal elements respectively (Table 1).

Secondly, we found that the limb connective tissue (quail marker) always remained localised within the limb (Fig. 2F) and formed the whole of humerus, the glenoid joint and the adjacent coracoid process (Supplementary Fig. S1). The connective tissue cells did not accompany the myogenic cells out of the limb into the trunk (n = 3/3). Thus only the muscle cells moved 'Out' into the trunk.

Importantly this work shows that the limb programme requires a contribution from axial sources (i.e. the connective tissues of the superficial girdle muscles).

Naïvity of girdle muscle precursors

These results suggest that superficial girdle muscles are patterned by local signals. Such a hypothesis would predict that any myogenic source should give rise to normal girdle muscles in the trunk. We carried out 4 sets of experiments to explore this avenue of thought, by transplanting either a leg bud, distal part of a leg bud, a tail bud or a branchial arch from a GFP chick in place of the wing bud of a wild-type recipient. Each manipulation gave rise to superficial girdle muscles (Figs. 3A–C). These results suggest that myogenic cells are patterned by local cues and that myogenic cells do not carry intrinsic positional information.

We explored this further by investigating the expression of *Hox* genes in myogenic cells following the development of superficial girdle muscles from the ectopic source. The hindlimb expresses *HoxB9* which is not found in the forelimb. Following transplantation of hindlimb to the forelimb position, we found that the grafted tissue gave rise to the superficial girdle muscles. However, intriguingly, the muscles maintained the expression of *HoxB9* (Fig. 3D). Therefore the myogenic cells are naïve with regard to their patterning potential despite retaining *Hox* gene expression.

Limb signals pattern axial somitic derivatives

Next we turned our attention to understanding the development of the skeletal elements of the pectoral girdle.

Our previous work has shown that a part of scapula which braces the limb to the axial skeleton (scapular blade in birds and medial scapular margin in mammals) has a unique origin. It develops from the dermomyotome of the somite by switching off its myogenic programme and by inducing cartilage development (Huang et al., 2000). This suggests that in order for a functional limb to develop, the limb programme must pattern not only lateral plate mesoderm but also induce a 'fate switch' in the somites.

We tested this line of thought by transplanting the prospective chick forelimb bud (somatopleura, HH12 (Stephens et al., 1989)) ectopically into the neck region (Fig. 4A). This transplantation gave rise to a complete ectopic limb (Fig. 4B) including the scapular blade and its associated rhomboid muscle (Figs. 4C and D).

This suggests that a single-unifying limb developmental programme controls the patterning of both the lateral plate mesoderm as well as indirectly the paraxial mesodermal compartments.

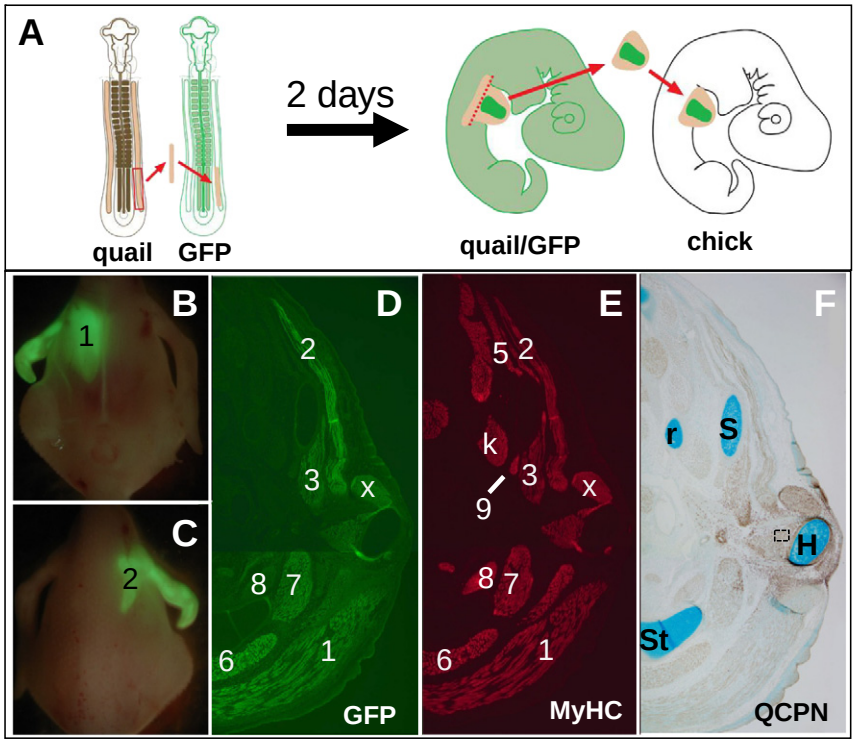

Fig. 2. Pectoral 'In–Out' principle followed only by myogenic cells and not by connective tissue. (A) Quail graft of lateral plate somatopleura lacking myogenic cells (HH15) was homotopically transplanted into GFP chick, which provided over 2 days GFP myogenic cells moving 'Into' the wing. Two days later at HH23 the compound chimeric limb bud was re-transplanted into wild-type chick. (B–F) 5 days later at HH35 the GFP labelled myogenic cells that moved 'Out' from the limb bud into the thorax (see in transverse section D), forming some girdle muscles (all muscles stained in E). No quail connective tissue cells (brown QCPN in parallel section F, cartilage in blue) were detected in the thorax. Quail connective tissue cells remained in the vicinity of the joint and its attaching muscles without reaching to the periphery of the muscles extending to the sternum, coracoid and scapula. GFP positive cells in the thorax overlapped with MyHC antibody staining (compare F and D). QCPN positive cells did not localise into the muscle fibers (dotted rectangle enlarged in Supplementary Fig. S2). *Muscle identification*: *1—pectoralis*, *2—latissimus dorsi*, *3—scapulohumeralis posterior*, *6—supracoracoideus*, *7—coracobrachialis posterior*, *8—subcoracoideus*; *5—rhomboidei*, *9—serratus superficialis anterior*; *x—triceps brachii* (*limb muscle*), *k—scalenus cranialis* (*neck muscle*); *skeletal cartilages: H—humerus*, *r—rib*, *S—scapula*, *St—sternum.*

Tbx5 is expressed in the thorax

We next investigated the molecular regulation of limb development with the aim of identifying a gene that controls not only the distal/appendicular region of the limb but also the tissues that brace the limb to the body.

Our search identified *Tbx5* as a good candidate as a master regulator of limb development. *Tbx5* expression was previously described as restricted to the forelimb (Isaac et al., 1998; Logan et al., 1998).

Our in-situ hybridisation analysis shows that *Tbx5* expression is also present in the proximal girdle region — the superficial tissues of the thorax, where the muscles bracing the limb to the trunk will be localised, and in the ventral midline region of the thorax where the sternum develops (Figs. 5A and B). Notably expression did not extend to the dorsal midline (Fig. 5C′) where the somites provide myogenic and cartilage cells for the medial scapular border (Valasek et al., 2010) and where latissimus dorsi will attach. This pattern was conserved between birds and mammals (Figs. 5A–C).

Tbx5 requirement for mammalian pectoral girdle development

To gain evidence for our hypothesis that *Tbx5* acts as a regulator of the entire limb, we examined the pectoral girdle of the mutant mice lacking *Tbx5* gene, which lack the appendicular forelimbs (Rallis et al., 2003). The analysis of the transitory region between the appendicular limb and the trunk — the pectoral girdle, has not been carried out yet.

Germline deletion of *Tbx5* is embryonically lethal by 10.0 dpc rendering it unusable for our studies. Therefore we conditionally deleted *Tbx5* in the lateral plate mesoderm using the Prx1–Cre line that permits viability until birth. The onset of Prx1–Cre activity is around 9.5 dpc (Logan et al., 2002) which is slightly after the onset of *Tbx5* expression (8.5 dpc) (Agarwal et al., 2003). Thus Tbx5 is only functional during a narrow early time window.

We found a complete absence not only of the appendicular forelimb, but also all skeletal elements of the girdle including the scapula, clavicle and the sternum (Rallis et al., 2003) (and our Supplementary Fig. S3). Girdle muscles developed to a limited extent and abnormally due to the absence of their skeletal attachment points (Figs. 6C and D). Intriguingly, the diaphragm of these animals only contained connective tissue but totally lacked skeletal muscle (Figs. 6E and F).

Thus the incomplete Tbx5 inactivation led to the absence of skeletal elements, while the girdle muscles were significantly affected.

Tbx5 requirement for pectoral girdle development in fish

We used the zebrafish as a developmental model firstly to examine our hypothesis in an evolutionary context. Classical comparative anatomical and paleontological studies (Romer, 1922) have proposed that abductor/adductor muscles of the fish are considered homologous to the pectoral girdle muscles of tetrapods, although detailed muscle homologies are not obvious (Diogo and Abdala, 2007). Secondly we took advantage of the fact that absence of tbx5 can be achieved in zebrafish as opposed to the partial condition delivered by the Prx1Cre/Tbx5 mouse line. Zebrafish have 2 copies of the *tbx5* gene. However *tbx5a* alone is expressed in the pectoral fin (Albalat et al., 2010) and the zebrafish *tbx5a*^{m21} mutant line (*heartstrings*) has a premature stop codon at residue 316 and is considered a null (Garrity et al., 2002).

All the skeletal elements of the pectoral fin (postcoracoid and scapulocoracoid processes and endochondral disc (Ahn et al., 2002;

Table 1
Superficial and deep girdle muscles. Categorisation of the girdle muscles into two developmental groups based on their lateral/medial developmental source (limb bud and myotomes respectively) and their initial insertion to the future skeletal elements. *Superficial girdle muscles* develop via 'In–Out' mechanism, therefore arriving to the girdle and the axial structures from the limb bud — from the lateral direction. Also their original (temporally earlier) attachments are lateral to the shoulder joint/coracoid process and only secondarily form attachments on the trunk. They develop from the ventral and dorsal pre-muscle masses (Sullivan, 1962, innervated by anterior and posterior divisions of the brachial plexus respectively (Williams et al., 1995)). *Deep girdle muscles* develop from local extensions of the myotomes, therefore arriving to the girdle from the medial direction. They are innervated by direct branches of the cervical roots. Their attachments are on girdle elements medial to the shoulder joint/coracoid process. The development of these muscles is non-migratory, independent of cMet signalling.

Avian girdle muscles		
Superficial		Deep
Ventral	Dorsal	
Pectorales	Latissimus dorsi	Rhomboidei
Supracoracoideus	Scapulohumerali	Serrati
Coracobrachialis	Anterior	Subscapularis
	Coracobrachialis posterior	
	Subcoracoideus	
	Deltoideus	
Mammalian girdle muscles		
Superficial		Deep
Ventral	Dorsal	
Pectorales (cutaneous maximus)	Latissimus dorsi	Rhomboidei
	Tereti	Levator scapulae
	Subscapularis	Serratus anterior
	Supraspinatus	
	Infraspinatus	
	Deltoideus	

Garrity et al., 2002)) were absent in the *heartstrings*. Importantly for this study we found that the abductor and adductor muscle groups were completely absent (Figs. 6G–L). Therefore all proximal — girdle musculature is absent in *heartstrings*. Additionally the cleithrum, to which the pectoral fin muscles partially attach, was absent (Fig. 6N) or severely hypoplastic (Ahn et al., 2002; Garrity et al., 2002). All other skeletal muscles were unaffected in *heartstrings*.

Discussion

Much work has been carried out to understand the development of the appendicular skeleton (the stylopod, zeugopod and the autopod) and the associated muscles. In contrast little is known about the development of the most proximal region — the pectoral girdle and its associated musculature in higher vertebrates. We describe the mechanism of formation of the girdle muscles and propose intriguing relationships between the limb programme, sternum and the diaphragm musculature, thus challenging the classical view of what is considered the trunk and the limb.

Pectoral girdle musculature deploys 'In–Out' mechanism

We have recently found that the cloacal sphincter muscles develop through a two-stage process; firstly myogenic cells migrate into the hindlimb bud but then extend out from the leg towards the ventral midline (Valasek et al., 2005) — a process called the 'In–Out' mechanism (Evans et al., 2006). Here we present evolutionarily conserved evidence for deployment of the 'In–Out' mechanism during the formation of the pectoral and latissimus dorsi muscles thereby providing experimental proof for suggestions made in previous studies (Beresford et al., 1978; Grim, 1971; Nagashima et al., 2009; Sullivan, 1962). A distinguishing feature between the 'In–Out' mechanism in the two regions is that the cloacal/perineal musculature loses connection with the hindlimb, while pectorals and latissimus dorsi muscles maintain attachment with the forelimb for functional reasons.

The initial movement of cells into the limb followed by a return into the trunk is reflected by the trajectory of the nerves that innervate the pectoral (medial and lateral pectoral nerves) and latissimus groups (thoracodorsal nerve). Their axons pass from the cervical spinal segments into the brachial plexus towards the axilla and then project back towards the ventral and dorsal midline respectively (Williams et al., 1995).

Trunk connective tissue do not display 'In–Out' characteristics

We found that the connective tissue of the limb bud does not undergo the 'In–Out' process like the muscle cells but instead remains localised to the transplanted limb. Therefore the muscle patterning information for the 'In–Out' cells returning to the trunk is derived from local sources and not from the limb. The nature of the patterning information is sufficient to support the formation of pectoral and latissimus dorsi muscles irrespective of the myogenic origin. We show that myogenic cells from a tail bud, branchial arch or a distal limb bud can be induced to move into the trunk to form the pectoral and latissimus dorsi muscles. Indeed, the molecular information carried by the myogenic cells is not important for the 'Out' phase of muscle development. We show that hind limb muscle cells that express *HoxB9* (Cohn et al., 1997) continue to do so in the trunk unlike the cells that normally form the pectoral and latissimus dorsi complex. Our studies suggest that in the context of the 'In–Out' mechanism the muscle cells carry no positional information but instead rely on patterning information from local connective tissue.

Three modes of forelimb muscle development

We propose three modes of muscle development in the forelimb programme: 1) Classical migration from the somites to the limb bud for all the muscles of the distal limb. 2) 'In–Out' process for the superficial girdle muscles (e.g. pectoral and latissimus dorsi). 3) Simple myotomal extension for the deep girdle muscles (e.g. serratus anterior, rhomboids) (Starck, 1982).

It has been proposed (Haines and Currie, 2001) that the chondrichthyan situation of epithelial extensions of somites populating the fins is representative of the primitive condition in vertebrate evolution. The Pax3/cMet/Lbx1 mechanism of migratory myogenic cells populating avian, mammalian and even some teleost limb buds is a derived mechanism. The existence of both modes of limb bud myogenic colonisation (epithelial extension and migratory myogenic cells), and also mixtures of these modes in some amphibians and reptiles (Galis, 2001) may be interpreted as supporting this view. Correlations of the two modes and the respective roles of the genes involved in limb myogenesis (e.g. Pax3 and Pax7, cMet, Lbx1) within appropriate primitive actinopterygian and sarcopterygian taxa are required to provide a clearer picture of the evolutionary history of limb muscularization.

Deep/superficial girdle muscles

We propose a nomenclature of the pectoral girdle muscles as "deep" and "superficial" which not only reflects their anatomical position, but mainly their medial/lateral developmental tissue source (myotome/limb), mechanism of development (myotomal extension/'In–Out' process) and also their original attachments medially and laterally to the shoulder joint/coracoid process, respectively (Table 1).

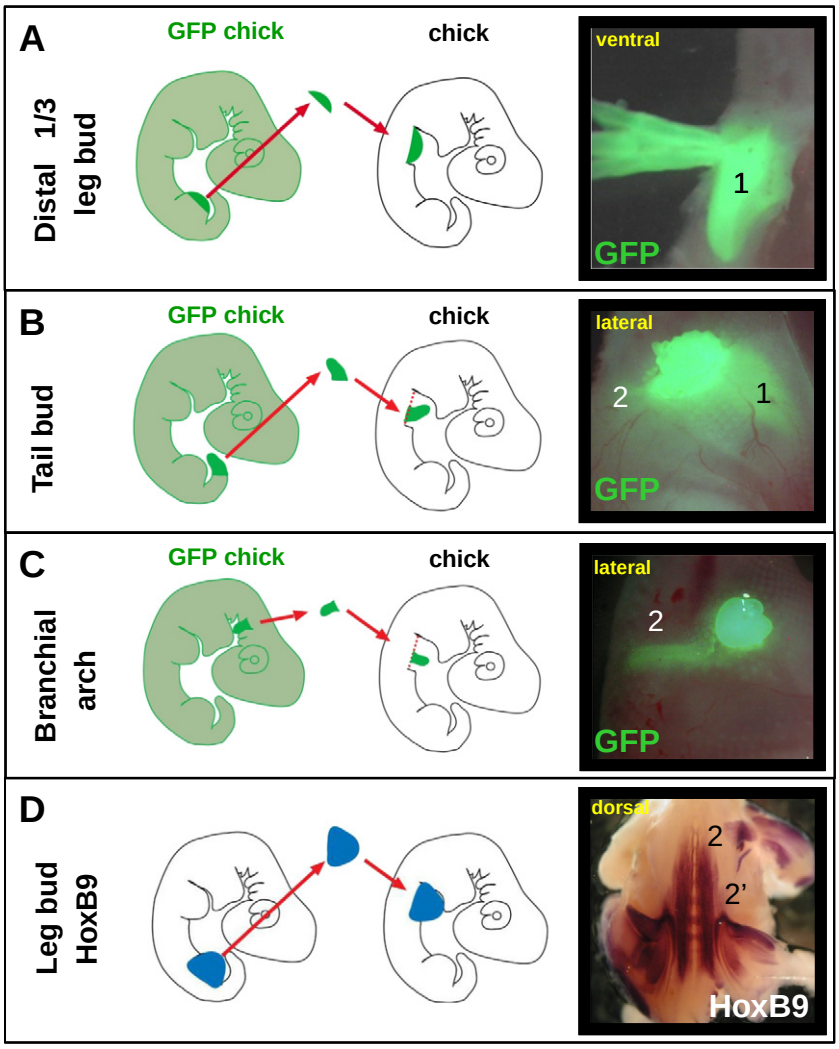

Fig. 3. Myogenic cells are patterned by local cues. (A, B, C) Transplantation of a GFP-chick tissue (HH23) in the place of a complete wing bud at HH23 resulted in GFP-positive superficial girdle muscles at HH35. The transplants were (A) a distal third of a leg bud (n = 2/2), (B) a tail bud (n = 2/2) and (C) a branchial arch (n = 3/3). The size of the GFP-tissue transplant determined the number and amount of GFP-musculature. (D) Leg bud tissue transplanted in place of a wing bud (n = 3/3) retains its original *HoxB9* expression despite forming forelimb girdle muscles as revealed by in-situ hybridisation at HH35. *Muscle names*: *1—pectoralis*, *2—latissimus dorsi* (*2—pars cranialis*, *2′ pars caudalis*).

Prunotto et al.(2004) described a puzzling presence of some girdle muscles in *cMet* null and *Splotch (Pax3* mutant*)* mice while other limb and girdle muscles were absent. Our study offers mechanistic explanation of their findings. All superficial girdle muscles are absent in *cMet* null or *Pax3* mutant (*Splotch*) (Prunotto et al., 2004), because the 'In–Out' mechanism relies in its first migratory phase on the cMet/HGF(SF) mediated migration. This includes the absent cutaneous maximus muscle which develops from the pectoral muscle anlage. The spino- and acromio-trapezius muscles are non-migratory head muscles innervated by the accessory XIth nerve, therefore they are not affected by *cMet* mutation. We have previously shown that the deep girdle muscles (serratus anterior, rhomboids and levator scapulae) are not affected by the *cMet* mutation (Valasek et al., 2010).

Forelimb programme patterns beyond the forelimb

Previous studies have demonstrated that the limb developmental programme is initiated by signals originating from axial structures (Saito et al., 2002; Stephens et al., 1989). A later reciprocal event occurs with the limb field signalling back to the axial somites to release myogenic cells and also to bring about a fate switch in the dermomyotomal cells which will form part of the scapula. We showed that the scapula can be induced from somites in the cervical region in the chick. This is particularly noteworthy since this territory is not able to form an ectopic limb in response to limb induction signals like FGF (Cohn et al., 1995). Only a complete ectopically transplanted limb field was able to induce the scapula.

This is in keeping with the evolution of the limb, where the appearance of the appendicular limb is followed by pectoral girdle development (DePalma, 2008) which braces the distal limb to the axial skeleton. In this context we showed that the limb programme was able to induce and recruit axial structures for its anchorage — the medial scapular border in mammals (Valasek et al., 2010) and the scapular blade in birds (Huang et al., 2000).

Tbx5 and definition of the forelimb programme

It is tempting to define the forelimb programme to comprise all cells that express *Tbx5*, based on the findings that the gene is transcribed not only in the limb bud but also in the superficial thorax where the superficial girdle muscles develop. However this is clearly not sufficient for instance for the somitic derivatives that give rise to

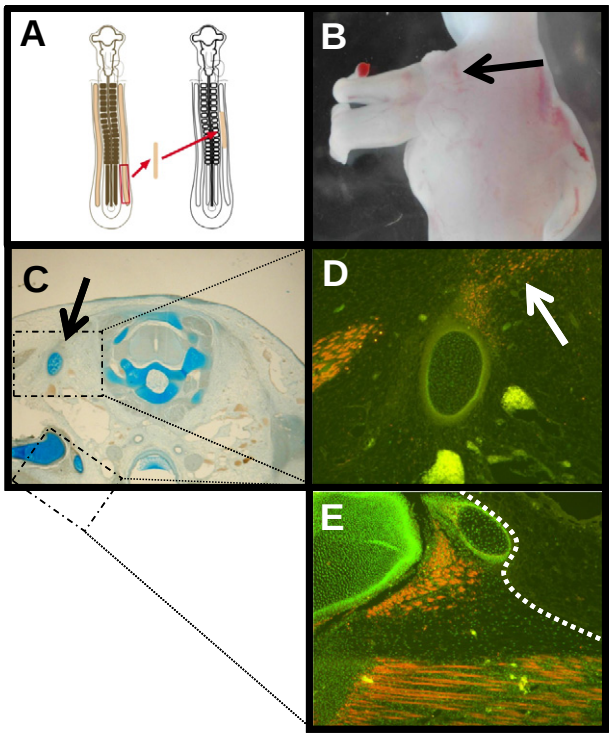

Fig. 4. Ectopic limb bud controls local somitic fate. (A) Somatopleura of a prospective quail limb bud (HH12) was transplanted into the future neck region of a chick. (B) HH33 embryo showing an ectopic quail wing (arrow) formed above the normal wing. (C) An ectopic scapular blade (arrow) is evident on the operated side in sections stained with Alcian blue for cartilage. No cartilage is present on the un-operated side. (D) Higher magnification of the inset image with fluorescent antibodies for muscles (red) and quail antigen (green QCPN) documents the formation of rhomboid muscles (arrow) dorso-medially from the ectopic scapular blade. Both structures are QCPN negative. (E) Second inset shows the ectopic quail humerus, clavicle and associated connective tissue positive for QCPN (green). The dotted line demarcates the extent of QCPN positive nuclei (green).

part of scapula as they do not express *Tbx5*. A better definition of the forelimb programme might be — all tissues that are limb-*Tbx5* dependent. By "limb-*Tbx5*" we refer to the fact that Tbx5 is also expressed in the heart, lens and genital region, probably by independent regulatory elements.

We describe *Tbx5* expression and function in the superficial trunk tissues, where the pectoral girdle and its muscles develop. The forelimb programme involves patterning tissues of various origins. These include the lateral plate mesoderm that gives rise to most of the limb skeleton and tendons (Chevallier et al., 1977; Christ et al., 1974b) and girdle skeleton as well as somitic dermomyotomal cells that form the limb muscles and the cartilage of the medial scapular border (scapular blade in birds) (Valasek et al., 2010). Indeed our analysis of the partial *Tbx5* inactivation in the mouse lateral plate mesoderm revealed abnormalities in all the tissues listed above. The absolute developmental dependence of the entire limb skeleton including the pectoral girdle and all of the associated girdle muscles was confirmed in the zebrafish *tbx5a*^{m21} (*heartstring*) null mutants (Garrity et al., 2002). We added the cleithrum as being dependent on the Tbx5 programme. This complements the findings of hypoplastic cleithrum following morpholino knockdown approach (Ahn et al., 2002). In few instances, we also observed hypoplastic cleithrum in our mutants, presumably representing hypomorphs.

The sternum is classically regarded as part of the axial skeleton closing the rib cage. However, it can be regarded as a mesenchymal condensation where the pectoral muscles attach. The sternum appears to be part of the Tbx5 limb programme as it is absent in our conditionally *Tbx5* inactivated mouse (Rallis et al., 2003; our Supplementary Fig. S3). This suggestion is supported by the development of the sternum from a paired ridge in the lateral plate mesoderm, like most of the limb and girdle skeleton (Chevallier et al., 1977; Christ et al., 1974a; Durland et al., 2008; Seno, 1961), and furthermore its development is independent (Chen, 1952; Fell, 1939) of the primaxial ribs (Durland et al., 2008). Evolutionarily the sternum appears in amphibians as their terrestrial lifestyle required strong ventral anchorage of the pectoral muscles. Amphibian ribs are short

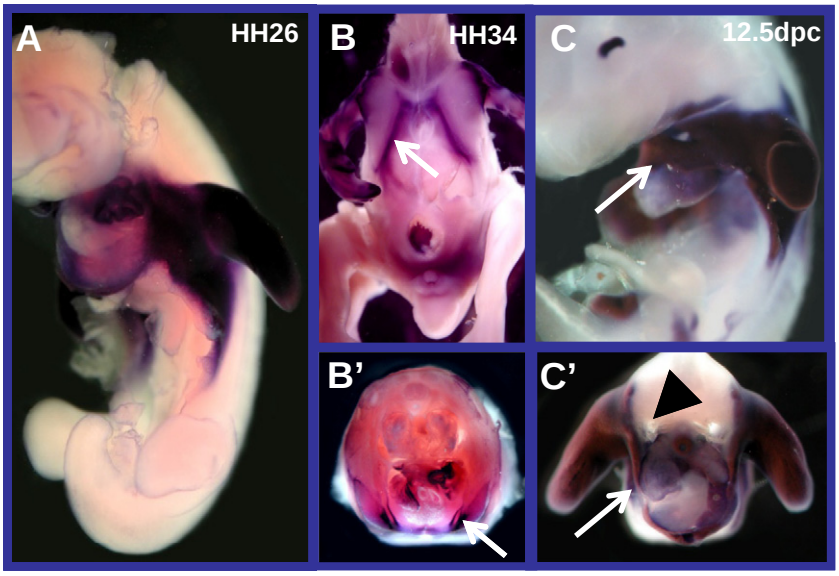

Fig. 5. *Tbx5* expression in thoracic wall in chick and mouse. (A–C) *Tbx5* is expressed in the limb bud and in the tissues of the superficial thorax in both chick (A, B) and mouse (C). (B—ventrolateral view) *Tbx5* expression in HH34 chick is restricted to the developing sternal anlagen (white arrow), while avoiding the actual cartilage (arrow in transverse view B′), as with other limb cartilages (not shown). (C—ventrolateral view) *Tbx5* in mouse is expressed not only in the tissue immediately adjacent to the limb base, but also covering the heart (arrow), where the sternum develops. Liver was removed. Arrowhead (in transverse view C′) denotes the growing axial structures — rib anlagen which are negative for *Tbx5* expression.

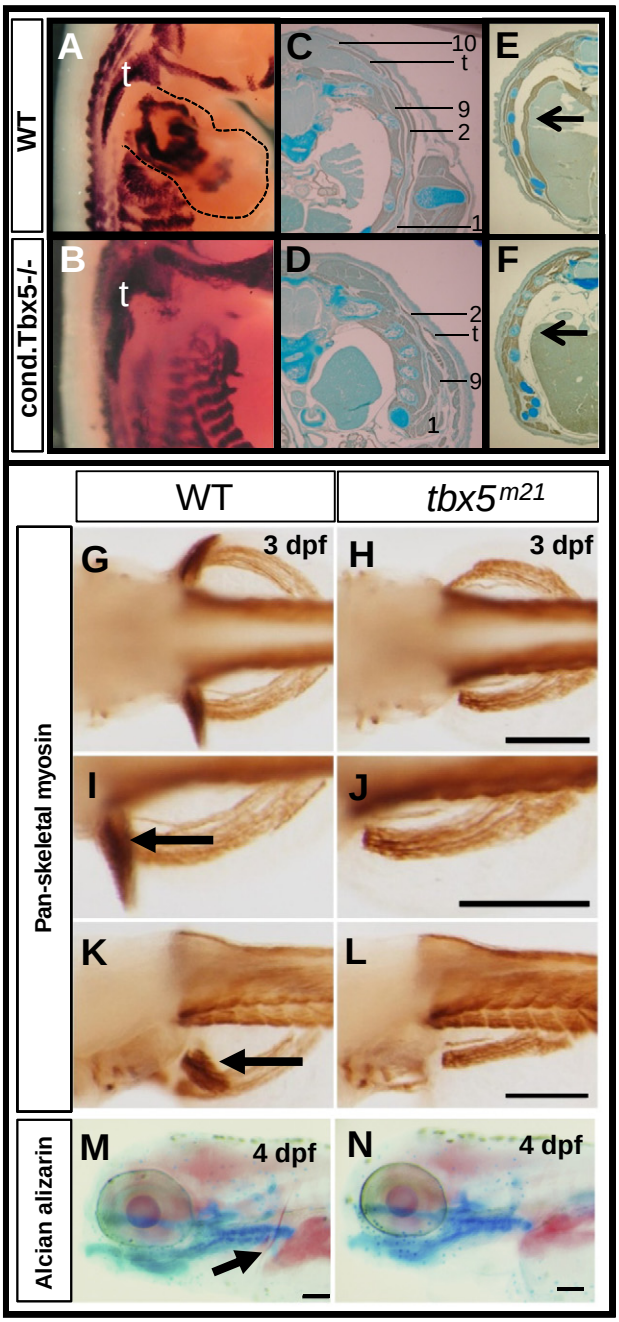

Fig. 6. *Tbx5* loss of function results in abnormal/absent pectoral girdle and diaphragm muscles. (A and B) *MyoD* expression in 12.5 dpc wild-type and conditionally *Tbx5* inactivated mouse embryos. The forelimb (outlined in A) is absent due to the lack of Tbx5 (B). Trapezius (t) — a neck muscle is preserved and intercostal muscles are more prominent (B). (C and D) 16.5 dpc transverse paraffin sections immunostained for muscle with Alcian blue counterstain document that the scapula, sternum (see Supplementary Fig. S3) and clavicle are also absent. Pectoral girdle muscles are abnormal (D), however analysis of serial sections revealed that no girdle muscle was completely absent. (E, F) Sections at more caudal level document that the diaphragm (arrow) is devoid of muscle in the conditional *Tbx5* inactivation (F). *Muscle identification: 1—pectoralis*, *2—latissimus dorsi*, *9—serratus anterior*, *10—cutaneous maximus*; *t—trapezius*. (G–N) In zebrafish *tbx5*^{m21} (*heartstring*) mutant, the pectoral fins (arrows in wt siblings) are absent including all fin muscles (adductors and abductors) with no traces of proximal fin musculature (Pan-MyHC(A4.1025) immuno staining with HRP). The cleithrum (arrow in M) to which they attach is also absent. Views: G–J dorsal K–N lateral. Scale bar 100 μm.

dorsal elements which do not reach the sternum (Fuchs, 1930; Howes, 1891). Animals which secondarily lost the pectoral appendage also lost the sternum (snakes, caecilians, and limbless lizards).

Partial development of girdle muscles in the partial Tbx5 inactivation

Complete *Tbx5* inactivation in zebrafish resulted in all limb/girdle muscles absent. However, in the conditional inactivation of *Tbx5*, the embryos failed to form any limb/girdle skeletal elements, yet some proximal girdle musculature formed to a limited extent. A possible explanation for this finding is that the earliest phase of Tbx5 expression is initiated prior to Cre mediated recombination (8.5–9.5 dpc) and permitted the development of the most proximal limb field. This was sufficient to support the recruitment of myogenic cells from the somites. Supporting evidence for this line of thought comes from a series of our limb bud ablations in chick before the 'In–Out' at HH20–21. All embryos (n = 12) failed to form the appendicular skeleton, yet in these cases the most proximal elements (scapula and sternum) have formed and displayed near-normal superficial girdle muscles (data not shown).

We suggest that in both experimental scenarios, although the distal appendicular field fails to develop, sufficient amount of the proximal portion of the limb has been specified to differing extents. In the case of the chick experiments, the ablation led to a normal girdle developing with its associated musculature. In the case of the mouse experiments, *Tbx5* ablation led to a more severe truncation of the limb, in this case encompassing the girdle. Yet this was still insufficient to remove the entire field thereby permitting the formation of the girdle muscles.

Diaphragm development and the limb programme

The musculature of the mammalian diaphragm develops from cervical myogenic precursors which briefly migrate through the lateral plate mesoderm and enter the connective tissue of pleuro-peritoneal folds which during further caudad differential growth of the pre-cervical tissues merge with the septum transversum (Clugston and Greer, 2007).

For the first time we provide evidence that the development of the muscular portion of the diaphragm is linked to the forelimb — through the Tbx5 programme. This link is supported by the segmental origin and innervation of these structures: the diaphragm's phrenic nerve in man is from C3 to C5, while the upper limb is innervated by C4–T2 (Williams et al., 1995). This overlap points towards the commonality of their migratory muscle precursors from the somites. Furthermore this situation is reminiscent of the cloacal sphincters providing caudal muscular closure of the abdominal cavity and being derived from the hind limb (Valasek et al., 2005). The cranial muscular closure of the abdominal cavity – the diaphragm – is similarly dependent on the forelimb.

The cloacal muscle precursors undergo the 'In–Out' via hindlimb (Valasek et al., 2005). However the development of the diaphragmatic muscle precursors is earlier and concomitant with the precursors for the limb bud so we propose they do not undergo the 'In–Out' via the limb bud, but instead they translocate from the cranial limb field directly into the pleuro-peritoneal fold. These structures express *SF/HGF* (Dietrich et al., 1999) allowing for the cMet-positive pre-cursors from the somites to migrate in the lateral plate mesodermal tissue. It is worth noting that we did not observe defects of the connective tissue of the diaphragm. Therefore it is likely that our conditional Tbx5 inactivation did not allow sufficient migration of myogenic cells in the cervical limb field to reach the pleuro-peritoneal fold.

It is worth noting, that although there is a link (via *Tbx5*) between the forelimb and the diaphragm muscle development, only mammals have a muscularized diaphragm. Thus, while the developmental/evolutionary origin of the muscularized diaphragm may depend on a forelimb genetic programme, the presence of forelimbs does not necessarily lead to muscularized diaphragms.

Conclusion

Our results explain the development of the superficial and deep girdle muscles of the forelimb and the influence of the forelimb field on the development of skeletal elements from lateral plate mesoderm and those that arise from the somites to form together the pectoral girdle.

Using *Tbx5* inactivation models we propose that a considerable amount of the trunk tissue should now be considered as a part of the limb developmental programme. This includes the connective tissue for the attachments of pectoral muscles — the sternum (and cleithrum in fish) and musculature of the mammalian diaphragm.

The authors declare no conflict of interests.

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